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Abstract
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Development and Structure of Bacterial Leaf Nodules in *Psychotria bacteriophila* Val. (Rubiaceae)

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The development and mature structure of bacterial leaf nodules in *Psychotria bacteriophila* were studied by using light and electron microscopy. Bacteria in mucilage surrounding the shoot apex pass through certain stomates in leaf primordia into the substomatal chamber. These chambers enlarge and become nodules as the young leaves grow out of the apical region. Surrounding mesophyll cells grow into each nodule and form a cellular reticulum whose interstices are occupied by bacteria. Each intrusive mesophyll cell wall is unusually thick and continually supplemented by vesicles originating from dictyosomes. The gram-negative bacteria are often surrounded by capsules. Nodule bacteria contain several crystal-like dense bodies. A population of normal, dividing, and degenerating bacteria is found in each nodule. Extensive membranes occur between the bacteria. A hypothesis is proposed to explain certain aspects of this obligate symbiotic relationship.

Bacterial nodules in roots are widely distributed among legumes, and have been reported from 10 genera of eight other families of angiosperms (16). Bacterial nodules in leaves are less common, but have been reported from approximately 370 species of five genera in the families Myrsinaceae and Rubiaceae. According to Humm (8), "Leaf nodules are typically small elevations of less than 2-mm diameter. They may be irregularly scattered over the surface of the leaf, located along the leaf margin only [characteristic of the Myrsinaceae], or present as two rows along each side of the midrib." Other reviews of leaf nodules are those of Boodle (1), Bremekamp (2), and Schaede (13).

Bacterial leaf nodules were first described from certain members of the Rubiaceae (19), and nodules in this family have received the most attention. Recent work has been concentrated on physiological aspects of the symbiosis in *Psychotria bacteriophila* (3, 14). Centifanto and Silver have shown that the bacteria can fix nitrogen, but that it is only of secondary importance. Bacteria-free seedlings supplied with nitrogen do not survive, which has led to speculation that hormones or other growth substances secreted by the bacteria are of primary importance.

Previous workers have only briefly outlined nodule development and mature structure at the light-microscope level. Ziegler (18) has shown electron micrographs at low magnification of the leaf nodule bacterium of *Pavetta zimmermanniana*, another member of the Rubiaceae. A detailed description correlating information from both the light- and electron-microscope levels is lacking. This report describes nodule initiation and development, bacterial morphology, and the close physical relation of bacteria to mesophyll cells within mature nodules, in *P. bacteriophila*. A hypothesis of bacteria-leaf cell interaction is also presented, based on this description.

MATERIALS AND METHODS

Greenhouse-grown clones from a plant of *P. bacteriophila* Val. (*P. punctata* Vatke) were used. Apical portions of terminal and axillary shoots were dissected out with a sterile razor blade. Nodular regions of immature and mature leaves were removed with a razor blade (for light microscopy) or punched out with a sterilized hypodermic needle (for electron microscopy).

Light microscopy. Material was fixed in CRAF III (12), dehydrated in an ethyl alcohol-xylene series, and embedded in Tissuemat (mp 53 C). Sections were cut 6 to 10 μ thick and stained with safranine-Chlorazol black E-fast green (7).

Electron microscopy. Material was fixed by the following methods: (i) 1% osmium tetroxide in a 0.1 M phosphate buffer, *p*H 7.25, at 4 C; (ii) unbuffered 2% potassium permanganate at 25 C; or, (iii) 3% glutaraldehyde in 0.1 M phosphate buffer, *p*H 7.25, for 12 hr at 4 C, followed by three 20-min washes in cold buffer, and then 1% osmium tetroxide in the

same buffer and temperature for 1 hr. Fixation (iii) gave the most consistent results. All material shown (Fig. 8–18) was fixed in this way. Fixed tissue was dehydrated in an ethyl alcohol-propylene oxide series and embedded in Epon 812 (9). Sections were cut with a diamond knife (500 to 800 A thick) on an ultramicrotome (LKB Instruments, Inc., Rockville, Md.) and stained for 10 min at 25 C with uranyl acetate dissolved in methanol (15). Observations were made on an RCA EMU-3F electron microscope.

Bacterial cultures. Work is in progress in the Department of Bacteriology at Iowa State University to identify the bacterium obtained from the plants used for this study. Cultures isolated from apical regions and nodules are being compared with cultures of leaf nodule bacteria studied by Centifanto and Silver (3).

RESULTS

Bacteria in the shoot apical region. Leaf arrangement is decussate in *P. bacteriophila*. After initiation, each pair of leaf primordia enlarges and arches over the shoot apex, forming a dome. As new pairs of leaves are initiated, a series of such enclosing domes results (Fig. 1). Maturing leaves expand and open out, so that the number of closed domes remains fairly constant. A pair of fused stipules develops early at the base of each young leaf, completing the enclosure of the shoot apex (Fig. 1). This pattern of shoot growth, characteristic of higher plants, allows the delicate shoot apex and young leaves to develop within a chamber which is presumably sealed from the outside.

In *P. bacteriophila*, numerous dendroid secretory trichomes are initiated from the upper epidermis of the fused pair of stipules. These trichomes develop rapidly and occupy most of the space between each stipule and the lower surface of the next youngest leaf primordium (Fig. 1). Each trichome contributes a mucilaginous secretion which fills the remaining space between leaf primordia, stipules, and the region above the shoot apex. A preliminary report of the development, structure, and function of these secretory trichomes is found elsewhere (6).

Bacteria are found in this constantly renewed pool of mucilage enclosed in the apical region of the shoot. Different taxa of bacteria have been isolated fron nodule-bearing plants, particularly from shoot apices and seeds. In the recent work of Centifanto and Silver (3), the gram-negative bacterium of *P. bacteriophila* was identified as *Klebsiella rubiacearum*.

Initiation and development of leaf nodules. Some bacteria, in a small amount of mucilage from the apical region, pass through the lower epidermis of young leaves via certain precociously formed stomatal pores. These bacteria occupy the substomatal chamber (Fig. 2, 3), which later enlarges greatly in volume and becomes the leaf nodule region (Fig. 4–7). Certain mesophyll cells bordering the developing nodule appear to grow into it (Fig. 4, 5), subsequently dividing to form numerous strands of cells (Fig. 6, 7).

Further growth appears to consist of multiplication of bacteria and continued intrusion of mesophyll cells into the nodule as the leaf expands. During this process, the leaf epidermis invaginates adjacent to the invaded stomatal pore, until the stomate finally occupies the bottom of a pit like depression (Fig. 3–7). At maturity, therefore, the nodule consists of an expanded region (substomatal chamber) traversed by numerous branched filaments of elongate mesophyll cells, with the interstices occupied by bacteria (Fig. 7).

In legume root nodules, bacteria are reported to stimulate polyploidy in infected cortical cells (17). Although we did not use chromosome squash techniques to see if this occurred in mesophyll cells in leaf nodules, we did observe that nodule mesophyll nuclei were similar in size and appearance to nuclei of other cells in the leaf (Fig. 2, 4, 5).

The mature nodule is bounded by mesophyll tissue, except where it is in contact with the invaginated epidermis. The two or three layers of mesophyll cells adjacent to the nodule appear to be compressed, without the conspicuous intercellular spaces characteristic of spongy mesophyll (Fig. 6, 7).

There is an average of 90 nodules per leaf based on a sample of five mature leaves taken from five plants used in this study. The exact number of nodules is proportional to the leaf area. Although a few nodules are inconspicuous, and a few are very conspicuous as a result of the fusion of two or three closely associated nodules, the great majority are from 0.4 to 0.8 mm in diameter, measured from surface view.

Ultrastructure of mature nodules. The mesophyll cells bordering each nodule lack intercellular spaces (Fig. 8), and each possesses a thick primary cell wall. This wall is progressively thinner in cells more distant from the edge of the nodule. Cells adjacent to the nodule possess the normal complement of organelles and inclusions and numerous starch granules occur in the chloroplasts, indicating normal photosynthetic activity (Fig. 8).

Within the nodule, intrusive mesophyll cells form a three-dimensional meshwork, with the individual cells generally elongate and somewhat distorted (Fig. 7, 9). Although almost completely surrounded by bacteria, the intrusive mesophyll cells appear to be healthy and functional. Numerous chloroplasts with starch granules are

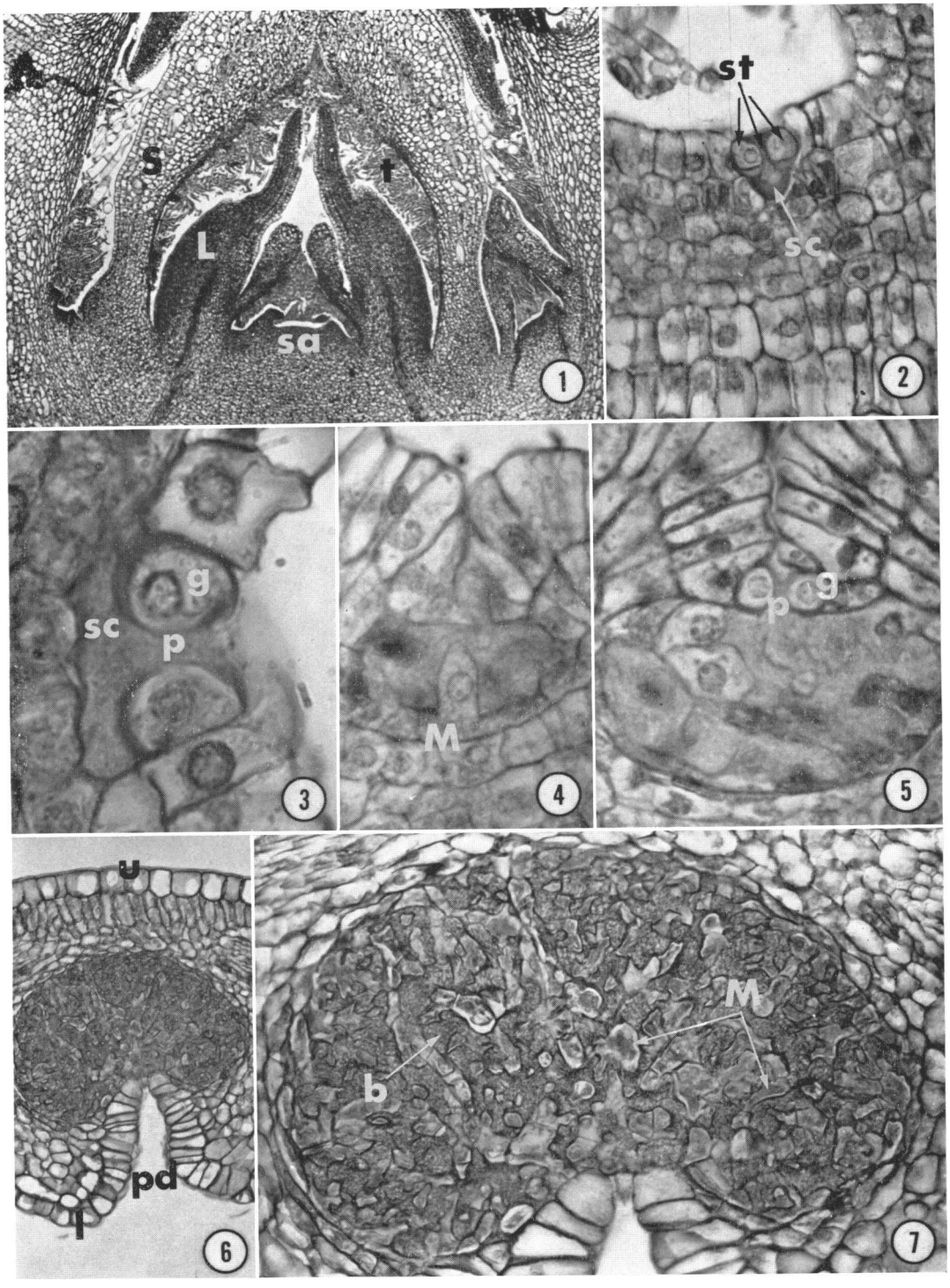

FIG. 1. *Median longitudinal section of apical region showing shoot apex (sa), leaf primordia (L), stipules (S), and trichomes (t). × 51.*

FIG. 2. *Transection of leaf primordium with precocious stomate (st) and small substomatal chamber (sc). × 510.*

FIG. 3. *Precocious stomate consisting of two guard cells (g), stoma (p), and substomatal chamber (sc) containing bacteria in mucilage. × 1,485.*

FIG. 4. *Young leaf nodule with surrounding mesophyll cells (M) beginning to intrude. × 638.*

FIG. 5. *Developing leaf nodule containing intruded mesophyll cells. Guard cells (g) and stoma (p) are still evident. × 680.*

FIG. 6. *Mature leaf nodule. Upper (u) and lower (l) epidermis and pitlike depression (pd) are visible. × 133.*

FIG. 7. *Mature leaf nodule with intrusive mesophyll cells (M) which form a network surrounded by bacteria (b). × 332.*

FIG. 8. *Edge of mature leaf nodule. Thick-walled mesophyll cells (M) are adjacent to bacteria (b).* × *7,800.*

found (Fig. 9) and cell walls, as in mesophyll cells bordering the nodule, are unusually thick (Fig. 9, 10).

There are no nodule bacteria that seem to be more than a few microns from a nodule mesophyll cell. Indeed, the nodule interior appears to consist of an extensive mesophyll framework completely surrounded by bacteria (Fig. 7). The bacteria are always intercellular and no dead or dying mesophyll cells were ever observed in mature or developing nodules. In legume root nodules, the bacteria are intracellular, forming colonies which are separated from the host-cell cytoplasm by a membrane or "envelope" (5).

Ultrastructure of the mesophyll cell wall. There is evidence that mesophyll cells in mature nodules are constantly depositing more cell wall material from within. Vesicles, containing electron-transparent material similar to that of the wall, occur throughout the cytoplasm; these appear to migrate to the plasmalemma, fuse with it, and deposit their contents outside (Fig. 10, 11). These vesicles appear to originate as blebs from dictyosomes, which are abundant in the mesophyll cells (Fig. 11). This mechnism of cell wall formation and deposition has been observed frequently by many investigators, but not in leaf mesophyll cells considered to be mature (11).

The outer part of the mesophyll cell wall is not sharply defined, and grades imperceptibly into the spaces between the adjacent bacteria (Fig. 9, 10). Most of the wall, however, appears to have a fibrillar structure (Fig. 10).

Bacterial morphology. We are in the process of isolating and specifically identifying bacteria from both the shoot apex and nodules. Our preliminary observations and comparison with the type specimen of Centifanto and Silver (3) are so far in agreement. At the ultrastructural level, bacteria from both the apical region and the nodule are nonflagellated and rodlike. The surface structures, consisting of a cell wall, plasma membrane, and intermediate layer, are typical of gram-negative bacteria (4). See Fig. 12 and 15 (insert).

Each bacterium in the extracellular mucilage of the apical region usually contains a conspicuous chromatic region and has a capsule surrounding the cell wall (Fig. 12). Several small electron-dense bodies are typically found in each bacterium. Bacteria in mature nodules are also rodlike, but sometimes lack a capsule. In addition, virtually every bacterium contains one or two large electron-dense bodies which appear crystalline in shape (Fig. 8, 9, 13, 14).

In each nodule, besides nondividing bacteria, constricted bacterial cells were commonly seen which appeared to be dividing (Fig. 15). In addition, there also occurred cells which were electron-dense and misshapen (Fig. 16, 18). These appeared to be undergoing degenerative changes. Associated with these degenerating bacteria, we observed a large part of surrounding membranes, some vesiclelike in profile, which were frequently quite extensive (Fig. 18). A smaller number of similar membranes were also found between apparently healthy bacterial cells (Fig. 9, 17)

DISCUSSION

Our results indicate that leaf-nodule bacteria in *P. bacteriophila* are confined to the extracellular mucilage surrounding the shoot apex, leaf primordia, and stipules, and within the expanded substomatal chamber that becomes the nodule. There are no other natural openings for bacterial entrance, and the lack of intercellular spaces in the mesophyll cells bordering the nodules makes it difficult to consider any systemic spread of nodule bacteria. We have other observations (*unpublished*) indicating that flower primordia, which are initiated as axillary buds, pick up mucilage as they grow through the apical shoot region and incorporate bacteria into the flowers and later into the seeds.

Because nodulated leaves are long-lived (*P. bacteriophila* is evergreen), and because the symbiosis has been shown to be obligate (3, 14), there must be a continuing exchange of certain necessary substances between bacteria and nodule mesophyll cells over a long period of time. Our frequent observations of dividing and degenerating bacteria within nodules indicate that at least a reasonably normal bacterial population continually exists there.

The extensive meshwork of intrusive mesophyll cells in the nodule, insuring close proximity to all bacteria, indicates that it is important that the bacteria not be a solid mass. We feel that this arrangement of mesophyll cells with thick, continuously supplemented cell walls, and the extensive occurrence of bacterial membranes, are morphological clues to the nature of this obligate symbiosis.

Our hypothesis, based at the moment only on morphological observations, is that the constantly renewed cell wall of the mesophyll cells bordering, and within, the nodule provides a convenient carbohydrate substrate for the bacteria. The membranes found between bacteria could be sites for enzymes capable of digesting the carbohydrates of the mesophyll cell wall. These membranes, furthermore, could also be involved in the transfer of whatever substance or substances the bacteria must contribute to the plant. Membranes from degenerating bacteria might be particularly important for the latter process. However, these

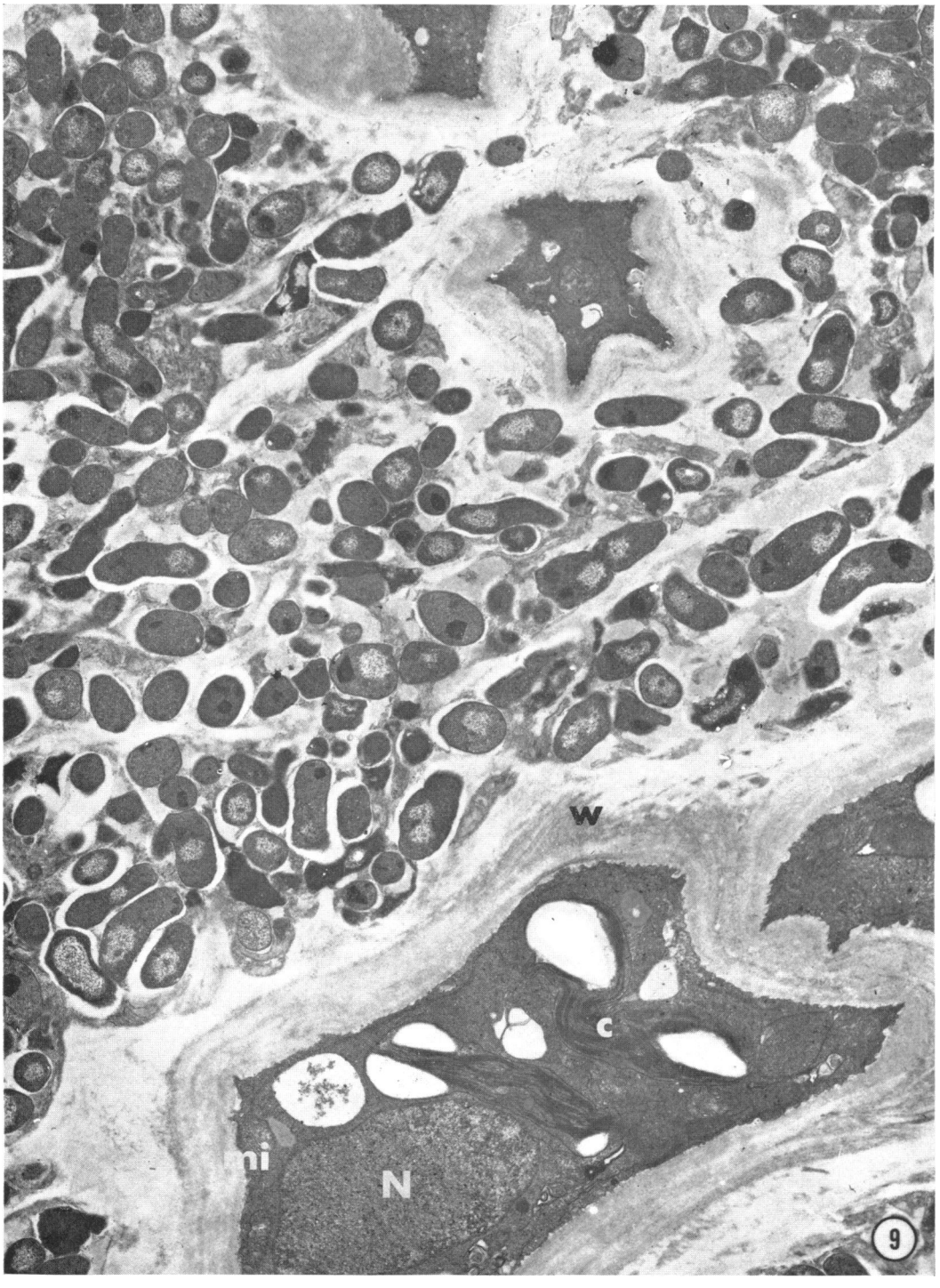

FIG. 9. *Interior of mature leaf nodule. Thick-walled (w), intrusive mesophyll cells contain nucleus (N), chloroplasts (c), and mitochondria (mi). These cells are surrounded by bacteria.* × *8,600.*

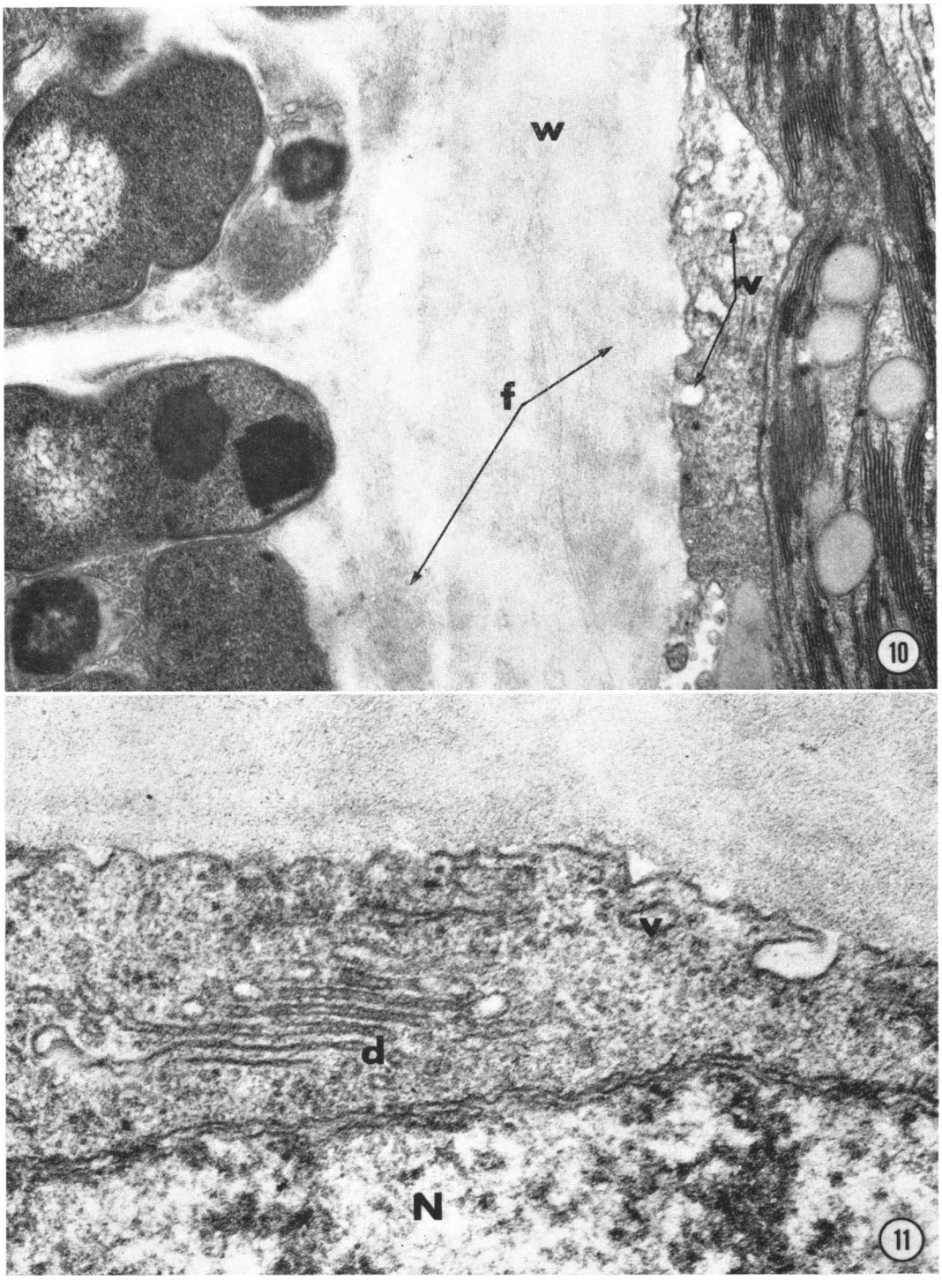

FIG. 10. *Interface of nodule mesophyll wall (w) between mesophyll cytoplasm and bacteria. Note fibrillar texture of wall (f), amorphous region of wall nearest bacteria, and Golgi vesicles (v) either adjacent to or fused with plasmalemma.* × *41,500.*

FIG. 11. *Portion of nodule mesophyll cell with dictyosome (d) and Golgi vesicles (v). Plasmalemma is irregular where vesicles have fused with it. Nucleus (N).* × *72,800.*

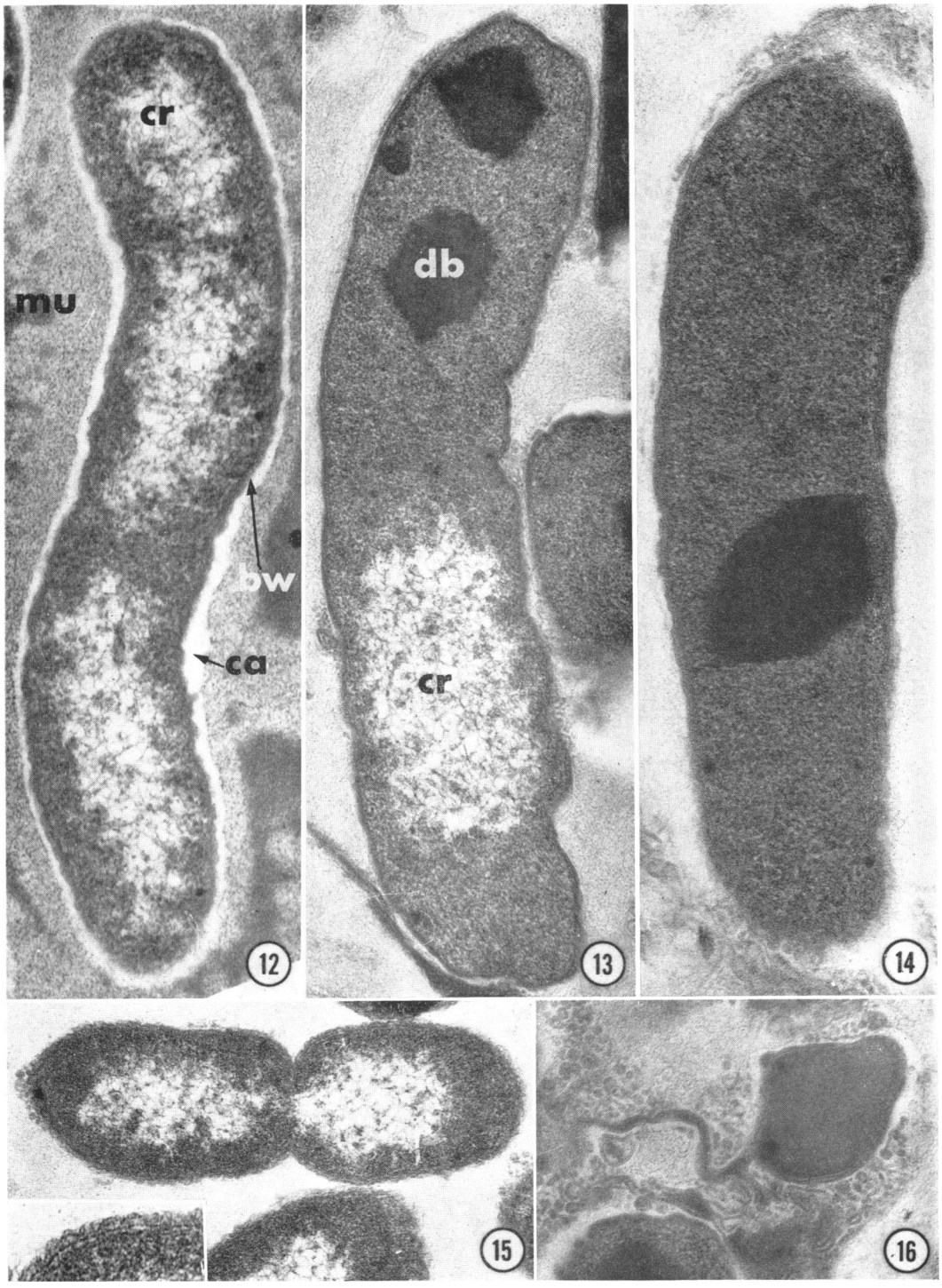

FIG. 12. *Bacterium in mucilage (mu) surrounding shoot apex. Note capsule (ca), bacterial wall (bw), and chromatic region (cr). × 46,200.*

FIG. 13. *Bacterium from mature leaf nodule. Note bacterial wall, chromatic region (cr), and two dense bodies (db). × 40,000.*

FIG. 14. *Bacterium from mature leaf nodule containing a dense body. × 54,400.*

FIG. 15. *Dividing bacterium from mature leaf nodule. × 40,000. Insert: enlarged portion of bacterial cell wall, plasma membrane, and intermediate layer.*

FIG. 16. *Degenerating bacterium from mature leaf nodule. Note proliferation of membranes and vesiclelike elements. × 52,000.*

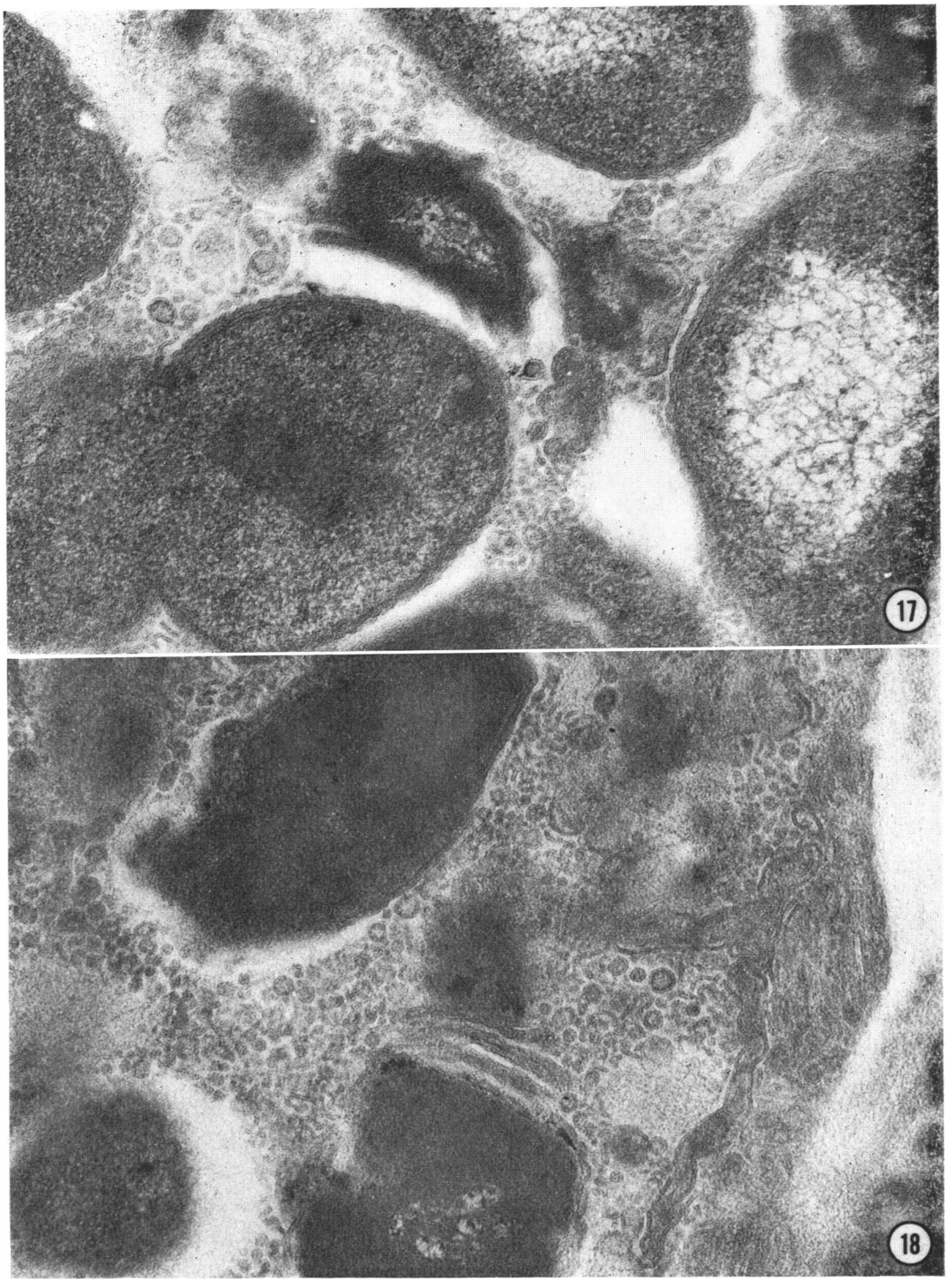

FIG. 17. *Portion of mature nodule showing proliferation of membranes between bacteria.* × *77,500.*
FIG. 18. *Portion of mature nodule showing degenerating bacteria. Membranes between bacteria are sheetlike and vesiclelike in profile.* × *77,500.*

membranes may merely represent excretory products, as has been shown for lysine-limited cultures of *Escherichia coli* (10). Although crystal-like inclusions in the bacteria of *P. bacteriophila* are common, our observation that they occur only in bacteria within nodules might indicate another factor of importance.

We have provided new morphological observations, and have advanced a working hypothesis concerning the leaf nodule symbiosis. To further our understanding, we plan similar light- and electron-microscope studies on other plants with leaf nodules. In addition, we plan to test our hypothesis by both in vivo and in vitro studies using histochemical, physiological, and other appropriate techniques.

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